

PTO/PCT Rec'd 5 SEP 2001

5

METHOD FOR INDUCING VIRAL RESISTANCE INTO A PLANT

10

Field of the invention

The present invention is related to a method for inducing viral resistance into a cell and a plant, especially BNYVV-resistance into a sugar beet cell and
15 plant.

Background of the invention and state of the art

The widespread viral disease of the sugar beet plant (*Beta vulgaris*) called Rhizomania is caused by a
20 benyvirus, the beet necrotic yellow vein virus (BNYVV) (23, 24) which is transmitted to the root of the beet by the soilborne fungus *Polymyxa betae* (25).

The disease significantly affects acreages where the sugar beet plant is grown for industrial use in
25 Europe, USA and Japan and is still in extension in several places in Western Europe (26, 27). As there exists no practical method to effectively control the spread of the virus at a large scale by chemical or physical means (28), neither in the plants nor in the soil, the main focus has
30 been to identify natural sources of resistance within the sugar beet germplasm and to develop by breeding, varieties of sugar beet plants expressing the resistance genes. A variety of such tolerance genes to the virus have been identified and, some have been successfully used in the
35 breeding of commercial sugar beet varieties (29, 30, 31).

Only the use of BNYVV-resistant or tolerant varieties will enable farmers to grow sugar beet plants in BNYVV-infected areas where the sugar beet plant is an essential component of the crop rotation and contributes
5 significantly to the grower's income.

A number of detailed studies have shown that a difference in susceptibility to the BNYVV-infection among sugar beet genotypes or varieties, generally reflect difference in the diffusion or translocation of the virus
10 in the root tissues (32).

However, there are still few reports which indicate clearly that the tolerance genes, even from differing sources of sugar beet germplasm or wild relatives germplasm (33), would provide distinct mechanisms of
15 resistance. Such a situation would represent a more manageable situation to design long lasting BNYVV-resistance strategies.

Since 1986, a number of reports and publications have described the use of isolated viral gene
20 sequences expressed in plants to confer a high level of tolerance against the virus or even to confer a broad spectrum type of resistance against a number of related viruses (34, 35, 36). One of the most documented viral resistance strategy based on genetic engineering, in many
25 cultivated species such as potato, squash, cucumber or tomato, is the use of the viral gene sequence which under the control of plant regulatory elements, encodes the coat-protein of the target virus (37).

However, for coat-protein mediated
30 resistance, the expression of a certain level of resistance in the transgenic plant might be attributed to different mechanisms such as RNA co-suppression and not necessarily to the production of the protein sequence.

In general, the virus sequence will be
35 transferred in an appropriate cell or tissue culture of the

plant species using an Agrobacterium mediated transformation system or a direct gene transfer method according to the constraints of the tissue culture or cell culture method which can be successfully applied in a given species. A whole plant will be regenerated and the expression of the transgene will be characterized.

Though sugar beet is known as a recalcitrant species in cell culture, limiting the extent of practical genetic engineering applications in that species, there are number of isolated reports of successful transformation and regeneration of whole plants (38). A few examples of engineering tolerance to the BNYVV by transforming and expressing the BNYVV coat-protein sequence in the sugar beet genome have also been published (39, WO91/13159) though they rarely report data on whole functional transgenic sugar beet plants (40). In particular, reports show limited data on the level of resistance observed in infected conditions with transgenic sugar beet plants transformed with a gene encoding a BNYVV coat-protein sequence (41, 42).

A complete technology package including a sugar beet transformation method and the use of the expression of the BNYVV coat-protein sequence as resistance source in the transgenic sugar beet plant obtained by said transformation method has been described in the Patent Application WO91/13159.

Based on the information published, it can not be concluded that the coat-protein mediated resistance mechanism provides any potential for conferring to the sugar beet plant a total immunity to the BNYVV-infection by inhibiting completely the virus multiplication and diffusion mechanisms. To identify a resistance mechanism which significantly blocks the spread of the virus at the early stage of the infection process would be a major step toward successfully developing such a transgenic

resistance. In addition, such resistance would diversify the mechanisms of resistance available.

Because the disease is shown to expand in many countries or areas, at a speed depending upon the combination of numerous local environmental and agricultural factors, there is a strong interest diversifying genetic resistance mechanisms which may, alone or in combination, confer a stable and long lasting resistance strategy in the current and future varieties of sugar beet plants which are grown for industrial use.

The genome of beet necrotic yellow vein benyvirus (BNYVV) consists of five plus-sense RNAs, two of which (RNAs 1 and 2) encode functions essential for infection of all plants while the other three (RNAs 3, 4 and 5) are implicated in vector-mediated infections of host plants (Beta macrocarpa, Beta vulgaris, Spinacear oleracea, Chenopodium quinoa, etc.) roots (1). Cell-to-cell movement of BNYVV is governed by a set of three successive, slightly overlapping viral genes on RNA 2 known as the triple gene block (TGB) (2), which encode the viral proteins P42, P13 and P15 (gene products are designated by their calculated M_r in kilodalton (3)).

In the following description, the TGB genes and the corresponding proteins will be identified by the following terms: TGB1, TGB2, TGB3 or by their encoded viral protein number P42, P13 and P15. TGB counterparts are present in other plant viruses and the characteristics of their TGB have allowed the classification of said viruses in two groups: the viruses of group I which include hordéiviruses, benyviruses, pecluviruses and pomoviruses and the viruses of group II represented by potexviruses and carlaviruses (4, 5, 6, 44).

For the viruses of group II, capsid protein is also involved in the cell-to-cell movement of viruses.

The development of a resistance to viral infections into a plant by blocking the cell-to-cell movement has been described for the potato viruses X (PVX) (45) and for the white clover mosaic virus (WC1MV) (46) in 5 Nicotiana benthamiana. These two viruses belong to the above-described group II. In both cases, various amino acids were replaced by Alanine in the hydrophilic part of the TGB sequence downstream of the N-terminal hydrophobic domain of said amino acid sequence. However, it was not 10 possible with said mutants to obtain total resistance, especially when a virus challenger concentration is increasing into the plant.

Aims of the invention

15 The present invention aims to provide a new method for introducing various viral resistances into a cell and a plant and the viral resistant cell and plant obtained.

A main aim of the invention is to provide a 20 new method for introducing BNYVV resistance into a cell and a plant and the BNYVV-resistant cell and plant, in particular a sugar beet cell and plant (Beta vulgaris ssp.), obtained.

25 Summary of the invention

The present invention provides the use of an alternative sequence of plant virus, especially the BNYVV, to obtain a high degree of tolerance to the viral infection, in particular to ensure a rapid and total 30 blocking of virus multiplication and diffusion mechanisms in a plant, especially in the sugar beet plant (Beta vulgaris), including fodder beet, Swiss chard and table beet, which may also be subject to this viral infection. Expression of the resistance will be obtained in transgenic 35 cell and plant, especially sugar beet cells and plants

produced by the transformation method subject to the Patent Application WO95/10178 or by other transformation methods based on Agrobacterium tumefaciens or direct gene transfer. Because of its high efficiency, the transformation method

5 as described in WO95/10178 enables the production of large numbers of transformed plants, especially sugar beet plants, and will be preferred to develop transgenic plants which may be analysed and characterized for their level of viral resistance, especially BNYVV Resistance, including

10 their field evaluation.

In the table 1 are represented viruses having a TGB2 sequence, the molecular weight of TGB2 of said viruses, their host and references.

Table 1

Virus	Size of TGB2 (kDa)	Host	Reference
GROUP I			
Beet necrotic yellow vein virus	13	beet	Bouzouba et al., <i>J. Gen. Virol.</i> 67, 1689-1700 (1986)
Barley stripe mosaic virus	14	barley	Gustafson et al., <i>Nucl. Acids Res.</i> 14, 3895-3909 (1986)
Potato mop top virus	13	potato	Scott et al., <i>J. Gen. Virol.</i> 75, 3561-3568 (1994)
Peanut clump virus	14	peanut	Herzog et al., <i>J. Gen. Virol.</i> 75, 3147-3155 (1994)
Beet soil-borne virus	13	sugar beet	Koenig et al., <i>Virology</i> 216, 202-207 (1996)
GROUP II			
Apple stem pitting virus	13	apple	Jelkman, <i>J. Gen. Virol.</i> 75, 1535-1542 (1994)
Blueberry scorch virus	12	blue- berry	Cavileer et al., <i>J. Gen. Virol.</i> 75, 711-720 (1994)
Potato virus M	12	potato	Zavriev et al., <i>J. Gen. Virol.</i> 72, 9-14 (1991)
White clover mosaic virus	13	clover	Forster et al., <i>Nucl. Acids Res.</i> 16, 291-303 (1988)
<i>Cymbidium</i> mosaic virus	14	orchid	Neo et al., <i>Plant Mol. Biol.</i> 18, 1027-1029 (1992)

The Inventors propose herewith a new method for providing resistance to plant viruses into a plant by

5 blocking virus multiplication and diffusion mechanisms into said plant, especially into its root tissue. In order to demonstrate said resistance, the Inventors describe hereafter the effect of the overexpression of TGB2 sequence alone or in combination upon BNYVV multiplication and

10 diffusion mechanism in plants of C. quinoa which are also

the hosts of the BNYVV virus and which could be more easily manipulated by the man skilled in the art.

It is known that BNYVV does not require synthesis of viral coat protein for production of local
5 lesions on leaves of hosts such as Chenopodium quinoa (7), indicating that virion formation is not required for cell-to-cell movement.

However, the manner in which the TGB components assist in the movement process is not understood
10 although computer-assisted sequence comparisons have detected characteristic conserved sequences which may provide clues to their function. Thus, the 5'-proximal TGB protein (TGB1) invariably contains a series of sequence motifs characteristic of an ATP/GTP-binding helicase while
15 the second protein (TGB2) always has two potentially membrane-spanning hydrophobic domains separated by a hydrophilic sequence which contains a highly conserved peptide motif of unknown significance (6).

So far, no example has been reported of a
20 virus of group I in which the three TGB members are arranged differently on the same RNA or are parcelled out to different genome RNAs, suggesting that their association in a particular order might be important in regulating their function.

25 The present invention concerns a method for inducing viral resistance to a virus of group I comprising the triple gene block (TGB2). Said viruses of group I comprise hordéiviruses, benyviruses, pecluviruses and pomoviruses, preferably viruses selected from the group
30 consisting of the beet necrotic yellow vein virus, the barley stripe mosaic virus, the potato mop top virus, the peanut clump virus and the beet soil-borne virus; said method comprises the following steps:

- preparing a nucleotide construct comprising a nucleotide
35 sequence corresponding to at least 70% of the wild-type

nucleotide sequence of TGB2 of said group I virus or its corresponding cDNA, being operably linked to one or more regulatory sequence(s) active in a plant,

- transforming a plant cell with the nucleotide construct,
- 5 and possibly
- regenerating the transgenic plant from the transformed plant cell.

Advantageously, the nucleotide sequence corresponding to at least 70% of the wild-type nucleotide sequence of TGB2 or its corresponding cDNA comprise the substitution of at least one amino acid into another different amino acid in the TGB2 wild-type sequence SEQ ID NO. 1 (Fig. 1). Preferably, the substitution of at least one amino acid into another different amino acid is made in regions rich in hydrophilic amino acids usually present at the surface of the corresponding protein in its native configuration. Preferably, a modification is made in the hydrophilic region of the wild-type sequence downstream the N-terminal hydrophobic domain and just upstream the conserved central domain.

According to a preferred embodiment of the present invention, said amino acids are each substituted by the amino acid Alanine.

Preferably, the plant or plant cell is a plant or plant cell which may be infected by the above-described virus and is preferably selected from the group consisting of potato, barley, peanut and sugar beet.

The present invention concerns also the obtained plant cell and transgenic (or transformed) plant (made of said plant cells) resistant to said viruses and comprising said nucleotide construct.

The Inventors have also discovered unexpectedly that it is possible to induce BNYVV-resistance into a plant by a method which comprises the following steps:

- 5 - preparing a nucleotide construct comprising a nucleotide sequence corresponding to at least 70%, preferably at least 80%, more preferably at least 90%, of the wild-type nucleotide sequence comprised between the nucleotides 3287 and 3643 of the 5' strand of the genomic or subgenomic wild-type RNA 2 of the BNYVV or its corresponding cDNA, being operably linked to one or more regulatory sequence(s) active in a plant,
- 10 - transforming a plant cell with said construct, and possibly
- regenerating a transgenic plant from the transformed plant cell.

The nucleotide sequence comprised between the nucleotides 3287 and 3643 of the 5' strand of the genomic or subgenomic RNA 2 encoding the P13 protein is described in the Fig. 1 (SEQ ID NO. 1). A preferred mutated nucleotide sequence and its corresponding mutated amino acid sequence are described in the following specification as SEQ ID NO. 3 (Fig. 2).

20 Another aspect of the present invention concerns a plant cell and a transgenic plant (made of said plant cells) resistant to BNYVV and comprising a nucleotide construct having a nucleotide sequence corresponding to at least 70%, preferably at least 80%, more preferably at least 90%, of the nucleotide sequence comprised between the nucleotides 3287 and 3643 of the 5' strand of the genomic or subgenomic wild-type RNA 2 of BNYVV or its corresponding cDNA, being operably linked to one or more regulatory sequence(s) active in the plant.

30 Preferably, said plant cell or transgenic plant (made of said plant cells) resistant to BNYVV is obtained by the method according to the invention.

The variants of the wild-type nucleotide sequence (SEQ ID NO. 1) comprise insertion, substitution or deletion of nucleotides encoding the same or different

35

amino acid(s) (see Fig. 2). Therefore, the present invention concerns also said variants of the nucleotide sequence of SEQ ID NO. 1, for example SEQ ID NO. 3, which present at least 70%, preferably at least 80%, more preferably at least 90%, homology with said nucleotide sequence and which are preferably able to hybridise to said nucleotide sequence in stringent or non-stringent conditions as described by Sambrook et al., §§ 9.47-9.51 in *Molecular Cloning : A Laboratory Manual*, Cold Spring Harbor, Laboratory Press, Cold Spring Harbor, New York (1989).

A nucleotide sequence corresponding to at least 70%, preferably at least 80%, more preferably at least 90%, of the nucleotide sequence comprised between the nucleotides 3287 and 3643 of the 5' strand of the genomic or subgenomic wild-type RNA 2 of BNYVV or its corresponding cDNA, is preferably a sequence comprising a substitution of at least one amino acid into another different amino acid in the wild-type RNA2 sequence of the BNYVV or its corresponding cDNA. Preferably said substitution is made in regions in which hydrophilic amino acids are usually present at the surface of the protein in its native configuration (47) as described in Fig. 2 (A = substitution by Alanine). Preferably, said substitution of one or more amino acids is a mutation which allows the substitution of one or more amino acids into one or more Alanine amino acids.

According to a preferred embodiment of the present invention, said nucleotide sequence is SEQ ID NO. 3.

Preferably, said sequences are also able to induce BNYVV resistance into a plant.

The terms "induce a viral resistance into a plant" mean inducing a possible reduction or a significant delay into the appearance of infection symptoms, virus

multiplication or its diffusion mechanisms into the plant, especially in the root tissues.

In Fig. 3 are represented results showing the capacity of a plant coinoculated with virus containing a replicon construct with the nucleotide sequence according to the invention, especially the sequence SEQ ID NO. 3, to inhibit the movement by BNYVV in *C. Quinoa*. The infectious factor of BNYVV is shown by the appearance of local lesions of leaves of said plant after co-inoculation of wild-type virus S12. Fig. 3 presents the number of local lesions upon leaves of a plant by a BNYVV S12 isolate (comprising RNA1 and RNA2) when co-inoculated with various replicons incorporating either mutated sequences including SEQ ID NO. 3 identified in Fig. 2 or a wild-type nucleotide sequence (T).

Eight days after said inoculation, the local lesions are identified. The results of three experiments show that the decreasing of said effect is mostly observed with the co-inoculation of the mutated sequence SEQ ID NO. 3 (up to 100% inhibition). This effect is not due to a possible blocking effect upon RNA1 and RNA2 replication, but the replicons according to the invention allow a blocking of the biochemical mechanisms involved in cell-to-cell movements by the infectious virus.

The regulatory sequence(s) of the nucleotide sequence according to the invention are promoter sequence(s) and terminator sequence(s) active into a plant.

The nucleotide construct may also include a selectable marker gene, which could be used to identify the transformed cell or plant and express the nucleotide construct according to the invention.

Preferably, the cell is a stomatal cell and the plant is a sugar beet (*Beta vulgaris ssp.*) made of said cells.

According to the invention, the promoter sequence is a constitutive or foreigner promoter sequence. Examples are 35S Cauliflower Mosaic Virus promoter sequence, polyubiquitin Arabidopsis thaliana promoter (43),
5 a promoter which is mainly active in root tissues such as the par promoter of the haemoglobin gene from Perosponia andersonii (Landsman et al., Mol. Gen. Genet. 214 : 68-73 (1988)) or a mixture thereof.

A last aspect of the present invention is
10 related to a transgenic plant tissue such as fruit, stem, root, tuber, seed of the transgenic plant according to the invention or a reproducible structure (preferably selected from the group consisting of calluses, buds or embryos) obtained from the transgenic plant or the cell according to
15 the invention.

The techniques of plant transformation, tissue culture and regeneration used in the method according to the invention are the ones well known by the person skilled in the art. Such techniques are preferably
20 the ones described in the International Patent Applications WO95/10178 or WO91/13159 corresponding to the European Patent Application EP-B-0517833, which are incorporated herein by reference. These techniques are preferably used for the preparation of transgenic sugar beets according to
25 the invention.

REFERENCES

1. Richards K.E. & Tamada T., *Annu. Rev. Phytopathol.* 30, pp. 291-313 (1992)
2. Gilmer D. et al., *Virology* 189, pp. 40-47 (1992)
- 5 3. Bouzoubaa S. et al., *J. Gen. Virol.* 68, pp. 615-626 (1987)
4. Herzog E. et al., *J. Gen. Virol.* 18, pp. 3147-3155 (1994)
5. Scott K. P. et al., *J. Gen. Virol.* 75, pp. 3561-3568 (1994)
- 10 6. Koonin E.V. & Dolja V.V., *Crit. Rev. Biochem. and Mol. Biol.* 28, pp. 375-430 (1993)
7. Schmitt C. et al., *Proc. Natl. Acad. Sci. USA.* 89, pp. 5715-5719 (1992)
- 15 8. Morozov S.Y. et al., *J. Gen. Virol.* 72, pp. 2039-2042 (1991)
22. Pelletier J. & Sonenberg N, *Nature* 334, pp. 320-325 (1988)
23. Tamada T. & Baba T., *Annals of the Phytopathological Society of Japan* 39, pp. 325-332 (1973)
- 20 24. Kuszala M. & Putz C., *Annals of Phytopathology* 9, pp. 435-446 (1977)
25. Tamada T., *CMI/AAB Description of Plants Viruses* 44, (1975)
- 25 26. Asher M.J.C., *Rhizomania In The sugar beet crop*, ed. D.A. Cooke and R.K. Scott, Chapman & Hall, London, pp. 312-338 (1993)
27. Richard-Molard M., *Rhizomanie In Institut français de la betterave industrielle. Compte-rendu des travaux effectués en 1994*, ITB, Paris pp. 225-229 (1995)
- 30 28. Henry C.M. et al, *Plant Pathology* 41, pp. 483-489 (1992)
29. Grassi G. et al., *Phytopath. Medit.* 28, pp. 131-139 (1989)

30. Merdinoglu D. et al., *Acad. Agric. Fr.* 79, n° 6, pp. 85-98 (1993)
31. Scolten O.E. et al., *Archives of Virology* 136, pp. 349-361 (1994)
- 5 32. Büttenr G. & Bürcky K., *Proceedings of the First Symposium of the International Working Group on Plant Viruses with Fungal Vectors*, Braunschweig Germany, August 21-24 (1990)
33. Whitney E.D., *Plant Disease* 73, pp. 287-289 (1989)
- 10 34. Powell A.P. et al., *Science* 232, pp. 738-743 (1986)
35. Fritchen J.H. & Beachy R.N., *Ann. Rev. Microbiol.* 47, pp. 739-763 (1993)
36. Wilson T.M.A., *Proc. Natl. Acad. Sci. USA* 90, pp. 3134-3141 (1993)
- 15 37. Gonsalves D. & Slightom J.L., *Seminars in Virology* 4, pp. 397-405 (1993)
38. D'Halluin K. et al., *Biotechnology* 10, pp. 309-314 (1992)
39. Kallerhof J. et al., *Plant Cell Reports* 9, pp. 224-228 (1990)
- 20 40. Ehlers U. et al., *Theoretical and Applied Genetic* 81, pp. 777-782 (1991)
41. Kraus J. et al., *Field performance of transgenic sugar beet plants expressing BNYVV coat protein plants*, Fourth International Congress of Plant Molecular Biology, Int. Soc. for Plant Molecular Biology, Amsterdam (1994)
- 25 42. Maiss E. et al., *Proceedings of the Third International Symposium on the Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms*, Monterey, pp. 129-139 (1994)
- 30 43. Norris et al., *Plant Molecular Biology* 21, pp. 895-906 (1993)
44. Solovyev et al., *Virology* 219, pp. 9-18 (1996)

45. Seppänen P. et al., *J. of General Virology* 78, pp. 1241-1246 (1997)
46. Beck et al., *Proc. Natl. Acad. Sci. USA* 91, pp. 10310-10314 (1994)
- 5 47. Cunningham et Wells, *Sciences* 244, pp. 1081-1085 (1989)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000
1001
1002
1003
1004
1005
1006
1007
1008
1009
1010
1011
1012
1013
1014
1015
1016
1017
1018
1019
1020
1021
1022
1023
1024
1025
1026
1027
1028
1029
1030
1031
1032
1033
1034
1035
1036
1037
1038
1039
1040
1041
1042
1043
1044
1045
1046
1047
1048
1049
1050
1051
1052
1053
1054
1055
1056
1057
1058
1059
1060
1061
1062
1063
1064
1065
1066
1067
1068
1069
1070
1071
1072
1073
1074
1075
1076
1077
1078
1079
1080
1081
1082
1083
1084
1085
1086
1087
1088
1089
1090
1091
1092
1093
1094
1095
1096
1097
1098
1099
1100
1101
1102
1103
1104
1105
1106
1107
1108
1109
1110
1111
1112
1113
1114
1115
1116
1117
1118
1119
1120
1121
1122
1123
1124
1125
1126
1127
1128
1129
1130
1131
1132
1133
1134
1135
1136
1137
1138
1139
1140
1141
1142
1143
1144
1145
1146
1147
1148
1149
1150
1151
1152
1153
1154
1155
1156
1157
1158
1159
1160
1161
1162
1163
1164
1165
1166
1167
1168
1169
1170
1171
1172
1173
1174
1175
1176
1177
1178
1179
1180
1181
1182
1183
1184
1185
1186
1187
1188
1189
1190
1191
1192
1193
1194
1195
1196
1197
1198
1199
1200
1201
1202
1203
1204
1205
1206
1207
1208
1209
1210
1211
1212
1213
1214
1215
1216
1217
1218
1219
1220
1221
1222
1223
1224
1225
1226
1227
1228
1229
1230
1231
1232
1233
1234
1235
1236
1237
1238
1239
1240
1241
1242
1243
1244
1245
1246
1247
1248
1249
1250
1251
1252
1253
1254
1255
1256
1257
1258
1259
1260
1261
1262
1263
1264
1265
1266
1267
1268
1269
1270
1271
1272
1273
1274
1275
1276
1277
1278
1279
1280
1281
1282
1283
1284
1285
1286
1287
1288
1289
1290
1291
1292
1293
1294
1295
1296
1297
1298
1299
1300
1301
1302
1303
1304
1305
1306
1307
1308
1309
1310
1311
1312
1313
1314
1315
1316
1317
1318
1319
1320
1321
1322
1323
1324
1325
1326
1327
1328
1329
1330
1331
1332
1333
1334
1335
1336
1337
1338
1339
1340
1341
1342
1343
1344
1345
1346
1347
1348
1349
1350
1351
1352
1353
1354
1355
1356
1357
1358
1359
1360
1361
1362
1363
1364
1365
1366
1367
1368
1369
1370
1371
1372
1373
1374
1375
1376
1377
1378
1379
1380
1381
1382
1383
1384
1385
1386
1387
1388
1389
1390
1391
1392
1393
1394
1395
1396
1397
1398
1399
1400
1401
1402
1403
1404
1405
1406
1407
1408
1409
1410
1411
1412
1413
1414
1415
1416
1417
1418
1419
1420
1421
1422
1423
1424
1425
1426
1427
1428
1429
1430
1431
1432
1433
1434
1435
1436
1437
1438
1439
1440
1441
1442
1443
1444
1445
1446
1447
1448
1449
1450
1451
1452
1453
1454
1455
1456
1457
1458
1459
1460
1461
1462
1463
1464
1465
1466
1467
1468
1469
1470
1471
1472
1473
1474
1475
1476
1477
1478
1479
1480
1481
1482
1483
1484
1485
1486
1487
1488
1489
1490
1491
1492
1493
1494
1495
1496
1497
1498
1499
1500
1501
1502
1503
1504
1505
1506
1507
1508
1509
1510
1511
1512
1513
1514
1515
1516
1517
1518
1519
1520
1521
1522
1523
1524
1525
1526
1527
1528
1529
1530
1531
1532
1533
1534
1535
1536
1537
1538
1539
1540
1541
1542
1543
1544
1545
1546
1547
1548
1549
1550
1551
1552
1553
1554
1555
1556
1557
1558
1559
1560
1561
1562
1563
1564
1565
1566
1567
1568
1569
1570
1571
1572
1573
1574
1575
1576
1577
1578
1579
1580
1581
1582
1583
1584
1585
1586
1587
1588
1589
1590
1591
1592
1593
1594
1595
1596
1597
1598
1599
1600
1601
1602
1603
1604
1605
1606
1607
1608
1609
1610
1611
1612
1613
1614
1615
1616
1617
1618
1619
1620
1621
1622
1623
1624
1625
1626
1627
1628
1629
1630
1631
1632
1633
1634
1635
1636
1637
1638
1639
1640
1641
1642
1643
1644
1645
1646
1647
1648
1649
1650
1651
1652
1653
1654
1655
1656
1657
1658
1659
1660
1661
1662
1663
1664
1665
1666
1667
1668
1669
1670
1671
1672
1673
1674
1675
1676
1677
1678
1679
1680
1681
1682
1683
1684
1685
1686
1687
1688
1689
1690
1691
1692
1693
1694
1695
1696
1697
1698
1699
1700
1701
1702
1703
1704
1705
1706
1707
1708
1709
1710
1711
1712
1713
1714
1715
1716
1717
1718
1719
1720
1721
1722
1723
1724
1725
1726
1727
1728
1729
1730
1731
1732
1733
1734
1735
1736
1737
1738
1739
1740
1741
1742
1743
1744
1745
1746
1747
1748
1749
1750
1751
1752
1753
1754
1755
1756
1757
1758
1759
1760
1761
1762
1763
1764
1765
1766
1767
1768
1769
1770
1771
1772
1773
1774
1775
1776
1777
1778
1779
1780
1781
1782
1783
1784
1785
1786
1787
1788
1789
1790
1791
1792
1793
1794
1795
1796
1797
1798
1799
1800
1801
1802
1803
1804
1805
1806
1807
1808
1809
1810
1811
1812
1813
1814
1815
1816
1817
1818
1819
1820
1821
1822
1823
1824
1825
1826
1827
1828
1829
1830
1831
1832
1833
1834
1835
1836
1837
1838
1839
1840
1841
1842
1843
1844
1845
1846
1847
1848
1849
1850
1851
1852
1853
1854
1855
1856
1857
1858
1859
1860
1861
1862
1863
1864
1865
1866
1867
1868
1869
1870
1871
1872
1873
1874
1875
1876
1877
1878
1879
1880
1881
1882
1883
1884
1885
1886
1887
1888
1889
1890
1891
1892
1893
1894
1895
1896
1897
1898
1899
1900
1901
1902
1903
1904
1905
1906
1907
1908
1909
1910
1911
1912
1913
1914
1915
1916
1917
1918
1919
1920
1921
1922
1923
1924
1925
1926
1927
1928
1929
1930
1931
1932
1933
1934
1935
1936
1937
1938
1939
1940
1941
1942
1943
1944
1945
1946
1947
1948
1949
1950
1951
1952
1953
1954
1955
1956
1957
1958
1959
1960
1961
1962
1963
1964
1965
1966
1967
1968
1969
1970
1971
1972
1973
1974
1975
1976
1977
1978
1979
1980
1981
1982
1983
1984
1985
1986
1987
1988
1989
1990
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020
2021
2022
2023
2024
2025
2026
2027
2028
2029
2030
2031
2032
2033
2034
2035
2036
2037
2038
2039
2040
2041
2042
2043
2044
2045
2046
2047
2048
2049
2050
2051
2052
2053
2054
2055
2056
2057
2058
2059
2060
2061
2062
2063
2064
2065
2066
2067
2068
2069
2070
2071
2072
2073
2074
2075
2076
2077
2078
2079
2080
2081
2082
2083
2084
2085
2086
2087
2088
2089
2090
2091
2092
2093
2094
2095
2096
2097
2098
2099
2100
2101
2102
2103
2104
2105
2106
2107
2108
2109
2110
2111
2112
2113
2114
2115
2116
2117
2118
2119
2120
2121
2122
2123
2124
2125
2126
2127
2128
2129
2130
2131
2132
2133
2134
2135
2136
2137
2138
2139
2140
2141
2142
2143
2144
2145
2146
2147
2148
2149
2150
2151
2152
2153
2154
2155
2156
2157
2158
2159
2160
2161
2162
2163
2164
2165
2166
2167
2168
2169
2170
2171
2172
2173
2174
2175
2176
2177
2178
2179
2180
2181
2182
2183
2184
2185
2186
2187
2188
2189
2190
2191
2192
2193
2194
2195